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Subject: TSNA Program Operational Plans for 1990.

OBJECTIVE(S): (1) To design a first generation laboratory model of a product by 1991 with MS TSNA (TSNA/mg TPM) delivery reduced 90% relative to the TPM-corrected TSNA delivery of a 1987 full-flavored, blended cigarette. (2) To design a second generation laboratory model of a product by 1993 with MS TSNA delivery (TSNA/mg TPM) delivery reduced 95% relative to the TPM-corrected TSNA delivery of a 1987 full-flavored, blended cigarette, utilizing technology based on a fundamental understanding of NA formation.

STRATEGIES:

REDUCTION OF MS TSNA BY REDUCING PREFORMED TSNA & PYROSYNTHETIC TSNA PRECURSORS IN FILLER

1. Reduce MS TSNA by selective removal of TSNA, amine precursors, and/or nitrosating agent precursors from filler.
2. Reduce MS TSNA by biochemical alteration(s) to tobacco leading to removal of alkaloid precursors of TSNA.

REDUCTION OF MS TSNA BY INHIBITING THE PYROSYNTHESIS OF TSNA

3. Reduce the levels of pyrosynthesized MS TSNA by incorporation into the cigarette design those aspects of oriental filler which result in an absence of significant TSNA pyrosynthesis from oriental tobacco.
4. Reduce the levels of pyrosynthesized MS TSNA by decreasing the reactivity to nitrosation of the amine precursors, or blocking reaction pathways which form nitrosating agents or which yield TSNA from the nitrosating agents.

REDUCTION OF MS TSNA BY ENHANCING DECOMPOSITION OF TSNA

5. Evaluate the enhancement of TSNA decomposition during smoking as a method for reducing TSNA delivery.

REDUCTION OF MS TSNA BY ALTERING PHYSICAL/CHEMICAL PARAMETERS OF CIGARETTES

6. Reduce the levels of pyrosynthesized MS TSNA by alterations in cigarette construction parameters.
7. Reduce the levels of pyrosynthesized MS TSNA by manipulation of filler salt content.
8. Reduce the levels of pyrosynthesized MS TSNA by manipulation of casings typically used in cigarettes but missing from the reference cigarette.

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TACTICS AND TIMETABLE:

1990

REDUCTION OF MS TSNA BY REMOVAL OF PREFORMED TSNA & TSNA PRECURSORS -

Selective Removal From Cured Filler Strategy:

Collaborate with ARD and PRD in development of SCFE
methodology capable of removing minor alkaloids from filler:
Prepare triethylamine bicarbonate and test for ability to
enable extraction of nornicotine using hexane
as solvent..... Nov. 1

Preparation of First Generation Low-TSNA Laboratory Model:

Finish development of Low-TSNA BCR reconstituted material.... Nov. 15
Incorporate Low-TSNA BCR reconstituted material into
extracted blend and evaluate..... Nov. 30
Add OrCEL to Low-TSNA blend and evaluate effect on MS TSNA... Dec. 15
Acquire appropriate additional data on these cigarettes..... Dec. 31

Biochemical Alterations to Tobacco Strategy:

Continue studies to confirm that the 60 kD band is PMT:
Continue ³H-SAM radiolabeling studies to label PMT:
Evaluate more sensitive methods for detecting label..... Dec. 31
Examine methods to optimize labeling..... Dec. 31
Substrate specificity studies..... Nov. 1
K_M Studies..... Dec. 1
Determine amino acid sequence of PMT:
Evaluate methods for cleaving PMT to obtain additional
sites for sequencing..... Dec. 31
Generate sample for further sequencing..... Dec. 31
Use the sequence to design oligonucleotide probes and have
these probes made..... Nov. 30
Use oligonucleotide probe to screen cDNA library..... begin
Nov. 30
Initiate studies involving PCR to amplify the gene coding PMT.. begin
Nov. 30
Develop methods for tobacco cell culture studies..... Dec, 31
Continue sequencing of overly expressed root clones..... as time
permits
Continue to provide purified PMT preps..... as needed
Initiate experiments relevant to next alkaloid biogenesis
pathway to be modified..... as time
permits

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Amine Precursor Strategy:

- Evaluate the role of unextracted nicotine (UN)
in TSNA pyrosynthesis by the following:
- Optimize Nagarse Type VII enzyme digestions of Bu marc
to separate Nic-Y and NN-Y from marc..... Dec. 31
 - Write SOP for the determination of different forms
of nicotine in tobacco and tobacco extracts..... Dec. 31
 - Isolate >4 kD Nic-X and <4 kD Nic-X by GPC from hot
water solubles (with 6912)..... Dec. 31
 - Evaluate relationship between Nic-Y and MS NNK by determining
Nic-Y and MS NNK in various water-extracted fillers..... Dec. 31
- NNK precursor studies:
- Pseudoxynicotine (PsON) method:
 - Evaluate SIM for GC/MSD..... Nov. 30
 - Decision point for method..... Nov. 30
 - Complete development..... Dec. 31
 - Develop plan to study existence of bound PsON, other
bound TSNA precursors, and bound TSNA..... Oct. 30
 - Develop plan to specifically address the reduction of NNK
in tobacco..... Dec. 31

REDUCTION OF MS TSNA BY INHIBITING PYROSYNTHESIS

Oriental Inhibitor Strategy:

- Complete evaluation of eight Or varieties:
- Data analysis using expert system software..... Dec. 15
 - Document study (including recommendations)..... Dec. 31
- Fractionate SC CO₂ Or filler extract to isolate potential inhibitor:
- Test fractions for inhibitory activity..... Nov. 30
 - If active fractions present, identify active components..... Dec. 15
 - Add Or inhibitor to Low-TSNA blend and evaluate..... Dec. 31

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ANALYTICAL METHODS DEVELOPMENT:

Develop new trapping system for aged/unaged SS smoke..... Dec. 15

Check new batch of ¹⁴C-NNN..... when
received

Investigate large Extrelut trap system for MS TSNA..... Dec. 31

Investigate SPE method for smoke/filler TSNA workup..... Dec. 31

Develop HPLC method (UV Vacancy Chromatog.) for
nitrate/nitrite with lower limit of detection than as time
currently available at PM R&D..... permits

SUPPORT OF OTHER PROGRAMS:

Design and carry out study of effects of cigarette paper on as time
nitrosamines in SS chamber..... permits

Project ART support..... ongoing

Support of other PM facilities..... ongoing

1991

Selective Removal From Cured Filler Strategy:

Optimize first generation low-TSNA model for TSNA reduction (2nd q).

Attempt to incorporate any design parameters determined by CSBW Program (3rd q).

Formulate plans for refined first generation low-TSNA lab model based on use of
a SCFE process designed to remove nicotine, preformed TSNA, and alkaloid
precursors of TSNA (4th q).

Biochemical Alterations to Tobacco Strategy:

Develop MPO activity assay (1st q).

Begin purification of MPO (1st q).

Use oligonucleotide probe to identify PMT gene (1st and 2nd q).

Develop 2D gel analysis capabilities in-house (2nd q).

Use expression vectors to confirm the PMT gene (3rd q).

Make anti-sense construct of PMT gene (begin 4th q).

Prepare vector(s) for insertion of anti-sense constructs (begin 4th q).

Transform tobacco cells with anti-sense construct of PMT (begin 4th q).

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1991 (Continued)

Amine Precursor Strategy:

Apply both forms of Nic-X to BW and evaluate as precursors for MS NNK (2nd q).

Identify Nic-X and Nic-Y (3rd q).

Develop smoke collection for analysis of PsON (2nd q).

Carry out a study of potential TSNA and their precursors which are chemically bound to soluble and insoluble tobacco components (3rd q).

Investigate removal of bound TSNA (if present) and their precursors by enzyme digestion followed by extraction (4th q).

Nitrosating Agent Strategy:

Initiate studies of role of NO and nicotine in combined gas stream to test for gas phase reaction & mechanism of NNK release (2nd q).

Develop methodology to test flash heating of TPM and filler to determine if TSNA precursors for pyrosynthetic type reactions are present in TPM and filler and to define time/temperature window for reaction (2nd q).

Test flash heating of TPM and filler to determine if TSNA precursors for pyrosynthetic type reactions are present in TPM and filler and to define time/temperature window for reaction (3rd q).

Finalize results from study on NO and nicotine in nitrosation and document (4th q).

Manipulation of Casings Strategy:

Plan and implement study to determine effect of casings typically used in cigarettes but missing from reference cigarettes (3rd q).

Support of Other Programs:

Project ART Support.

Support of other PM facilities.

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1992-1993

Amine Precursor, Nitrosating Agent, and Oriental Inhibitor Strategies:

Utilize enzyme treatment combined with extraction techniques to produce fillers yielding low MS TSNA. Incorporate pyrosynthesis inhibitors, NO scavengers, NNK pyrosynthesis inhibitors (if available) and antioxidants into the extracted fillers to yield a second generation low-TSNA model.

Biochemical Alterations to Tobacco Strategy:

Screen tobacco cells for reduced PMT and/or nicotine expression.

Regenerate transformed plantlets; select those with lowest nicotine.

Transform tobacco cells with anti-sense construct of MPO.

Support of Other Programs:

Support of PM facilities.

1994

Transfer technology for low TSNA model.

Biochemical Alterations to Tobacco Strategy:

Further develop and test transformed tobacco plants.

Support of Other Programs:

Support of PM facilities

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RESOURCE ALLOCATIONS FOR 1990:

How are the personnel assigned to this program allocated?
(These allocations are for personnel in the BCR Division)

	Professionals	Technicians
Extraction of preformed TSNA and TSNA Precursor Strategy:	1.20	0.50
Biochemical Alteration of Tobacco Strategy:	9.25	1.00
Oriental Inhibitor Strategy:	1.30	0.30
Amine Precursor Strategy:	2.40	0.50
Nitrosating Agent Strategy:	0.90	0.50
Cigarette Construction Parameters Strategy:	0.30	0.20
TOTAL	15.35	3.00

TRANSFER OF TECHNOLOGY:

The target date for the first generation laboratory model of a reduced TSNA product is 1991. An interim technology based primarily on lab-scale solvent extraction will be utilized until SCFE methodology capable of removing minor alkaloids (now being investigated) can be developed. When the latter has been accomplished, the corresponding technology will be transferred to Development. At that time, companion technologies involved in preparation of the low-TSNA filler can also be transferred to Development if deemed desirable.

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